Gene family for an elicitor-induced sesquiterpene cyclase in tobacco

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ABSTRACT The initial step in the conversion of the isoprenoid intermediate farnesyl diphosphate to the sesquiterpene phytolalexin capsidiol in elicitor-treated tobacco tissues is catalyzed by an inducible sesquiterpene cyclase [5-epi-aristolochene synthase (EAS)]. Two independent cDNA clones (cEAS1 and cEAS2) encoding EAS were isolated from an elicitor-induced tobacco cDNA library by differential hybridization and subsequently were characterized by hybrid selection-in vitro translation. Insertion of cEAS1, a partial cDNA clone encoding 175 C-terminal amino acids, into an Escherichia coli expression vector resulted in accumulation of a fusion protein immunodetectable with EAS-specific polyclonal antibodies. The cDNA clones were used to isolate two full-length EAS genes that mapped 5 kilobases (kb) apart on one 15-kb genomic clone. The nucleotide sequences of the structural gene components were identical from 388 base pairs (bp) upstream of the transcription initiation site to 40 bp downstream of the translation termination codon, suggesting a relatively recent duplication event. The genes consist of 1479-bp open reading frames, each containing five introns and specifying 56,828-Da proteins. The N-terminal amino acid sequence deduced from the genomic clones was identical to the first 16 amino acids of the EAS protein identifiable by Edman degradation. RNA blot hybridization with cEAS1 demonstrated a mRNA induction time course consistent with the induction of the EAS mRNA translational activity with maximum levels 4-6 h after elicitation. EAS mRNA was not detected in control cells. DNA blot-hybridization analysis of genomic DNA revealed a copy number of ~12-15 for EAS-like genes in the tetraploid tobacco genome. The conservation of a putative allelic prenyl diphosphate binding motif is also discussed.

Sesquiterpenoids are a structurally diverse class of isoprenoids found in plants, some fungi, and bacteria, but not in vertebrates, which have important implications in plant-plant (1), plant-insect (2, 3), and plant-pathogen (4, 5) interactions. Despite an extensive appreciation for the structure-function relationships of plant sesquiterpenoids, the sesquiterpenoid biosynthetic pathway and its regulation are not well understood (4, 6). Although many enzymes of general isoprenoid biosynthesis have been measured in plants, few enzymes suspected of being rate-limiting have been identified (4, 6). Moreover, little is known about the mechanisms regulating the activity and absolute levels of plant isoprenoid biosynthetic enzymes with the exception of a diterpene cyclase (7). This is in contrast to the extensive molecular analysis of regulatory mechanisms controlling isoprenoid metabolism in mammalian systems (8).

Tobacco cell suspension cultures respond to treatment with elicitors such as fungal cell wall hydrolysates or cellulase by the de novo synthesis and secretion of antibiotic sesquiterpenoids, primarily the phytolalexin capsidiol (9, 10). Central to capsidiol biosynthesis is the induction of a sesquiterpene cyclase [5-epi-aristolochene synthase (EAS)] that catalyzes the cyclization of trans,trans-farnesyl diphosphate (FPP) to the bicyclic intermediate 5-epi-aristolochene (9). The induction of sesquiterpene cyclase enzyme activity and, hence, sesquiterpenoid biosynthesis has also been correlated with the suppression of squalene synthetase enzyme activity and consequent decline in sterol biosynthesis (9, 10). The induction of one enzyme and the suppression of the other are interpreted as an important regulatory mechanism controlling end-product formation, since the two enzymes are positioned at putative branch points in isoprenoid metabolism (9).

Tobacco EAS is a soluble, apparently cytoplasmic enzyme determined by a single gene family (9) that is expressed de novo in response to elicitation and that is constitutively active in the absence of elicitation in suspension cultures (11). Changes in the de novo synthesis rate of the EAS protein were also correlated with changes in the EAS mRNA translational activity. Further investigation of the regulation of isoprenoid metabolism and in particular the sesquiterpenoid branch pathway in plants will require detailed molecular analysis of the EAS gene(s), especially those sequences involved in transcriptional regulation.

MATERIALS AND METHODS

Cell Cultures, Elicitor Treatment, and Assays. Tobacco (Nicotiana tabacum L. cv KY14) cell suspension cultures in their rapid growth phase were treated with cellulase (Trichoderma viride, Type RS, Onozuka) as elicitor to a final concentration of 20 μg/ml (13). Control and elicitor-treated cells were collected by gentle vacuum filtration and used for measurements of EAS enzyme activity (12) and the level of EAS mRNA (14, 15).

Library Constructions and Screening. A cDNA library was constructed in pcDNAII (Invitrogen, San Diego) using poly(A)+ RNA from 4-h-elicitor-treated tobacco cells and was screened by differential hybridization (16) with radiola-belled first-strand cDNAs complementary to either control or elicitor-induced poly(A)+ RNAs. Clones exhibiting preferential hybridization to elicitor-specific probe were further characterized by the hybrid selection-in vitro translation-immunoprecipitation technique (17). A putative EAS cDNA (cEAS1) identified as such was used to isolate additional cDNA and genomic clones. A λEMBL3 genomic library, constructed from Mbo I partially digested tobacco (N.

Abbreviations: EAS, 5-epi-aristolochene synthase; IPTG, isopropyl 1-thio-β-D-galactoside; FPP, trans,trans-farnesyl diphosphate; TS, trichodiene synthase.

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tabacum L. cv NK326) hypnotol DNA (Clontech) was screened according to a standard protocol (15). Genomic DNA fragments were subsequently inserted into pBluescript KS(+) for further characterization (15).

**Isolation and Analysis of Nucleic Acids.** Total RNA was prepared by the guanidine thiocyanate/CsCl method (14), and poly(A)* RNA was isolated by oligo(dT)-cellulose chromatography. Total RNA (20 μg) was fractionated on 1.0% agarose gels containing formaldehyde and transferred to nylon membranes (15). RNA blot hybridizations were performed with radiolabeled DNA probes labeled with ^32P-dCTP (15) as probe at 42°C in 50% (vol/vol) formamide/5× SSPE at pH 8.0 (1× = 0.15 M NaCl/10 mM NaH2PO4/1 μM EDTA, pH 8.0)/1× Denhardt’s solution (0.02% polyvinylpyrrolidone/0.02% bovine serum albumin/0.02% Ficoll)/0.5% SDS/100 mg of denatured salmon sperm DNA per mL. Tobacco leaf genomic DNA was isolated (18), digested with various restriction endonucleases, electrophoresed on a 0.8% agarose gel, and transferred to nylon membranes. DNA blots were hybridized with random-primer-radioabeled (15) cEAS1 at 60°C in 0.25 M sodium phosphate buffer, pH 8.0/7.0% SDS/1% bovine serum albumin/1 mM EDTA. Both DNA and RNA blots were washed at 45°C, twice with 2× SSC/0.1% SDS and twice with 0.2× SSC/0.1% SDS (15) (1× SSC = 0.15 M NaCl/0.015 M sodium citrate, pH 7). Relative mRNA transcript levels were estimated from RNA blot autoradiograms with a video densitometer (MilliGen/Biosearch, Ann Arbor, MI).

**Fine Mapping of EAS Structural Genes.** Intron junctions were mapped by nucleotide sequence comparison of cDNA and genomic clones. A 5′ intron not covered by the partial cDNA clones was identified by using a cDNA fragment amplified by reverse transcription–polymerase chain reaction (RT-PCR) with primers specific to exonic sequences on either side of the intron. The RT reaction was catalyzed with Moloney murine leukemia virus reverse transcriptase at 42°C by using elicitor-induced poly(A)* RNA primed with the antisense 3′ oligonucleotide (5′-TGATGCTTTCATAGTGAAAGC-3′). First-strand cDNAs were amplified by 35 cycles of the PCR after addition of the sense 5′ primer (5′-AGAAATTGTAGAGATTTTGG-3′) and adjustment of reaction conditions for Taq DNA polymerase. The purified 222-base pair (bp) PCR product was sequenced directly by using the same primers.

The sequence alignment of the initiation site was identified by S1 nuclease protection analysis performed with elicitor-induced poly(A)* RNA and PCR-amplified double-stranded DNA probe (15). The PCR primers were specific to genomic sequences located 106 bp downstream (5′-CCTTTATCT-CATCAATTTC3′) and 282 bp upstream (5′-TTGCACAC-TATGAAAGAG-3′) of the ATG codon corresponding to the predicted N-terminal methionine. The primers were 5′-end-labeled with T4 polynucleotide kinase and [γ-^32P]ATP prior to use in the PCR.

**Nucleotide and Amino Acid Sequencing.** Sequencing of single- and double-stranded DNAs was by the dideoxynucleotide chain-termination method (19) with Sequenase (United States Biochemical) or an automated fluorescence-based system (Applied Biosystems) or both. Both strands of insert DNAs were sequenced from their ends using T7 and T3 primers. In addition, 20-mer oligonucleotides synthesized according to obtained sequence information were used directly as primers for further sequencing.

N-terminal amino acids of the EAS protein were obtained by Edman degradation on a pulse-liquid-phase sequenator (Applied Biosystems). Purified EAS protein was cleaved with cyanogen bromide, and the products were separated by SDS/PAGE and then transferred to a poly(vinylidene difluoride) membrane. A Coomassie blue-staining band of ~20 kDa was used for sequencing.

**Expression of Partial EAS cDNA in Escherichia coli.** Two oligonucleotides (5′-GGGATCTTAAAGGAATTGTAAGAATT-3′ and 5′-GGGAAATTCGGTCTAGT- TAGGAAGTCCA-3′) specific to cEAS1 and designed to include 5′ BamHI and 3′ EcoRI restriction sites, respectively, were used to prime the PCR with cEAS1 as template. The amplified 700-bp product was inserted into the BamHI/EcoRI sites of the expression vector pGEX-2T (Pharmacia). E. coli JM101 cells harboring the pGEX-EAS1 construct were grown in Luria–Bertani medium to OD₆₀₀ = 0.6, and expression of the fusion protein was induced by addition of isopropyl 1-thio-β-D-galactoside ( IPTG) to a final concentration of 1 mM. Cells were collected 1 h after addition of IPTG. Proteins were solubilized in SDS sample buffer, separated by SDS/PAGE, transferred to nitrocellulose, and immunodecorated as described by Leary et al. (20).

**RESULTS**

**Isolation and Characterization of Clones.** Differential hybridization (15) and hybrid selection–in vitro translation (17) techniques were used to isolate a cDNA clone complementary to the EAS mRNA. The in vitro translational activity of EAS mRNA has been detected only in RNA preparations from elicitor-treated cells, suggesting the presence of EAS transcripts exclusively in induced mRNA populations (12). Hence, a cDNA library was prepared to poly(A)* RNA from 4-h elicitor-induced tobacco cell cultures. Clones exhibiting preferential hybridization to the radiolabeled first-strand cDNA probe synthesized from the induced mRNA pool, versus the probe prepared to control mRNAs, were subsequently used for RNA blot hybridizations. Twelve of 46 clones hybridized to an elicitor-induced mRNA with an induction time course consistent with the EAS mRNA translational activity and did not hybridize to control mRNAs. Nine clones that did not cross-hybridize to one another were further characterized by hybrid selection–in vitro translation. One cDNA clone (cEAS1) hybrid-selected a mRNA from the population of mRNAs isolated from elicitor-treated cells which, when translated in vitro in the presence of [35S]methionine, directed the synthesis of an ~60-kDa protein immunoprecipitable with EAS-specific antibodies. The cDNA library was rescreened with cEAS1 as probe, and a second cDNA (cEAS2) was recovered (Fig. 1C). Nucleotide sequence analysis showed that the two partial cDNAs were independent transcripts.

A tobacco genomic library in λEMBL3 was screened by using cEAS1, and eight independent genomic clones were isolated. Restriction endonuclease analysis suggested that each genomic clone represented a different gene. One 15-kb genomic clone was selected for further characterization because the cDNA probe hybridized to two locations on the DNA fragment separated by ~6 kb (Fig. 1A). Subsequent analysis indicated that the two regions contained 2517 bp of identical nucleotide sequence, which were identified as duplicated EAS genes (gEAS3 and gEAS4). The nucleotide and predicted amino acid sequences of gEAS3 are shown in Fig. 2. Each gene consists of an open reading frame of 1479 bp divided among six exonic segments and interrupted by five introns with a range in size of 75–155 bp (Figs. 1B and 2).

The two structural genes (gEAS3 and gEAS4) exhibited 100% nucleotide sequence identity in both coding and non-coding regions, from 388 bp upstream of the transcription-initiation site to 40 bp downstream of the transcription-termination codon. The partial cDNAs (cEAS1 and cEAS2) each exhibited 94% and 95% identity to the structural genes and 95% and 93% identity relative to each other at the nucleotide and amino acid levels, respectively, to a position 40 bp downstream of the translation-termination codon. The nucleotide sequences of all four genes beyond the conserved 40 bp 3′ to
the translation stop codon were completely divergent (Fig. 1). A putative "TATA" box was identified 25 bp upstream of the transcription start site (Fig. 2). Potential polyadenylation signals were also observed in the region of gEAS3' to the stop codon. Similar signals were detected in the divergent region of gEAS4.

The predicted translation product initiated at the first in-frame ATG following the transcription start site has a molecular mass of 56.8 kDa, consistent with the value of 60 kDa determined for the EAS protein by SDS/PAGE (11). Additional evidence that the gene encodes the previously purified EAS protein (11) was obtained by comparing the empirically determined N-terminal amino acid sequence of EAS with the predicted sequence as shown in Fig. 2. Sixteen of the first 18 amino acids were identifiable by Edman degradation and corresponded with the predicted amino acid at each position.

Expression of EAS Polypeptide in E. coli. PCR amplification of the partial cDNA insert of cEAS1 using primers with specifically adapted 5' BamHI and 3' EcoRI restriction sites allowed for ligation of the fragment into the open reading frame of the human glutathione S-transferase gene contained on the E. coli expression vector pGEX-2T. The 700-bp PCR product contains a termination codon 525 bp from the introduced 5' BamHI restriction site and encodes 175 amino acids at the C terminus of the EAS protein. Cell homogenates of E. coli JM101 transformed with the recombinant plasmid pGEX-EAS1 and induced with IPTG contained a 45-kDa fusion protein immunodetectable with mouse polyclonal antibodies prepared to purified EAS (Fig. 3). Only trace amounts of the polypeptide were detectable in uninduced cultures. Untransformed cultures and those transformed with the nonrecombinant pGEX-2T plasmid did not produce any proteins detectable with the EAS-specific antibodies.

Induction of EAS mRNA in Response to Elicitor. The induction of EAS mRNA was monitored directly by hybridization.
ization of the radiolabeled cDNA insert from cEAS1 to total RNA isolated from control and celluspule-treated cultures at various times after addition of elicitor (Fig. 4A). No EAS transcripts were detected in control RNA. Elicitor treatment resulted in the appearance of detectable levels of EAS mRNA in less than 4 h, with a maximum occurring 6 h after elicitation, followed by a subsequent gradual decline (Fig. 4B). EAS enzyme activity was also induced in elicitor-treated cells, but the rate of increase in enzyme activity was that of the EAS mRNA. Although the level of EAS mRNA was transient with a maximum at 6 h and a return to control levels within 24 h, EAS enzyme activity was detected for more than 48 h after elicitation.

**Gene Family for EAS in Tobacco.** Tobacco genomic DNA digested with either EcoRI or HindIII and probed with radiolabeled cEAS1 revealed 12 and 11 bands, respectively, suggesting the existence of numerous EAS-like genes (Fig. 5). In contrast, only one predominant band appeared in Xba I digests (Fig. 5). The size of the fragment (800 bp) is consistent with the separation distance of the two Xba I sites on the EAS genomic clones (Fig. 1). The predominant Xba I band was estimonimetry to be 15 times more intense than the average band intensity in HindIII- or EcoRI-digested DNA, confirming a copy number of ~12–15. The single intense Xba I band also suggests a high degree of structural similarity among all EAS genes in tobacco. Secondary bands apparent in the Xba I-digested DNA may represent hybridization of the cEAS1 probe to genomic fragments containing most of the 3' untranslated gene-specific sequences. The divergent sequences on the four EAS cDNA and genomic clones begin 120 bp downstream of the 3' Xba I site, a feature apparently common to all EAS genes (Fig. 1).

**DISCUSSION**

We report the isolation and characterization of genes encoding the sesquiterpene cyclase EAS from tobacco. The gene for a fungal sesquiterpene cyclase, trichodiene synthase (TS), from *Fusarium sporotrichioides* has also been characterized (21). Although both enzymes use FPP as the sole substrate, comparison of the predicted amino acid sequences using the FASTDB search algorithm did not reveal significant overall homology between these proteins or any other entry in the Protein Identification Resource (May 1992) or SwissProt (May 1992) protein sequence data bases. However, several other proteins that use FPP or related prenyl substrates contain aspartate-rich consensus sequences proposed as binding sites for the charged head group of these substrates (22). Sequences in both EAS and TS containing the putative consensus motif Asp-Asp-Xaa-Xaa-Asp were aligned with the proposed allylic prenyl diphasphate binding domains of FPP synthase (23) and squalene synthetase (24) from yeast (Fig. 6). EAS and TS exhibit 33% identity relative to each other and 33% and 23% identity, respectively, relative to FPP synthase among 18 amino acid residues that include the Asp-Asp-Xaa-Xaa-Asp motif. The apparent dissimilarity of the squalene synthetase domain has been suggested to reflect the unique head-to-tail (1'–4) condensation catalyzed by squalene synthetase, in contrast to the more common head-to-tail (1'–4) condensations catalyzed by other prenyl binding proteins (24). The moderate sequence conservation of the EAS and TS aspartate-rich domains relative to the proposed consensus sequence of FPP synthase may indicate a similar substrate binding function. Additional experimentation, including site-directed mutagenesis, is necessary to further evaluate the function of this domain.

Transcriptional control of EAS enzyme activity was suggested previously by thiouridine-labeling experiments, which measured the in vitro translational activity for EAS mRNA in *de novo* synthesized RNA populations from elicitor-treated and control cells (12). EAS mRNA translational activity was detected only in RNAs from elicited cells with a maximum 4–6 h after addition of elicitor. The induction patterns for the steady-state level of EAS transcripts (Fig. 4) and the EAS mRNA translational activity (12) are identical. The minimal time lag for the induction of EAS enzyme activity subsequent to the increase in mRNA level (Fig. 4B) is also in agreement.

**Fig. 4.** RNA gel blot analysis of EAS gene expression in elicitor-treated tobacco cell suspension cultures. Total RNAs from cells at various times after treatment were separated on a denaturing agarose gel, transferred to a nylon membrane, and probed with cEAS1. (A) Autoradiogram of the RNA blot. (B) Time course of elicitor-induced EAS mRNA accumulation (a) and enzyme activity (c). EAS mRNA levels were quantified by video densitometry. EAS enzyme activity was measured in a fraction of the cells used for RNA extraction. Maximum specific EAS enzyme activity was 16.3 nmol of product per h per mg of protein.

**Fig. 5.** DNA gel blot analysis of the EAS-like genes in tobacco. Genomic DNA (5 μg) was digested with EcoRI, HindIII, or Xba I; separated by electrophoresis; transferred to a nylon membrane; and probed with cEAS1 at high stringency.

**Fig. 6.** Alignment of a proposed allylic prenyl diphasphate binding motif (22) in EAS, TS, yeast FPP synthase (Y-FPS), and yeast squalene synthetase (Y-SS). Amino acids asparagine (N) and aspartate (D) are considered a conservative substitution (24).
with earlier results, which showed that changes in the de novo synthesis rate of the EAS protein correlate with changes in mRNA translational activity (12). The results reported herein together with those reported previously (12) show that the induction of EAS enzyme activity in elicitor-treated tobacco cell suspension cultures is primarily regulated by transcriptional control of the EAS gene.

The rapid transcriptional induction of EAS in tobacco after elicitor treatment is consistent with reports on the expression pattern of genes encoding other key enzymes from diverse phytoalexin biosynthetic pathways. For example, in castor bean (Ricinus communis L.), the single step conversion of geranylgeranyl diphasphate, another allylic diphasphate isoprenoid intermediate, to the diterpenoid casbane is catalyzed by the elicitor-inducible and transcriptionally regulated enzyme casbane synthetase. A partial casbane synthetase cDNA was used to demonstrate maximum accumulation of transcripts in castor bean seedlings 6 h after treatment with a pectic elicitor (7). In addition, the accumulation of antibacterial furanocoumarins by members of the Leguminosae is dependent on the transcriptional induction of two important enzymes (25), phenylalanine ammonia lyase and chalcone synthase. Recently, the biosynthesis of cytotoxic benzophenanthridine alkaloids in the Papaveraceae and Fumariaceae was also correlated with elicitor-mediated transcriptional regulation of the berberine bridge enzyme (26).

The detection of multiple bands in EcoRI- or HindIII-digested genomic DNA (Fig. 3), the isolation of numerous independent genomic and cDNA clones (Fig. 1), and the existence of two genes (geAS3 and geAS4) that map only 5 kb apart (Fig. 1) suggest that the EAS genes in tobacco are a partially clustered gene family comprised of ~12–15 members. The duplication of genes for important rate-determining enzymes of phytoalexin biosynthesis has also been observed for phenylalanine ammonia lyase (27, 28) and chalcone synthase (29). Although the gene for chalcone synthase is present in only a single copy in parsley (Petroselinum crispum) and other nonlegume species (30), a gene family of six to eight partially clustered members has been identified in French bean (Phaseolus vulgaris) (28) and another legume, soybean (Glycine max) (25). Several of the chalcone synthase genes are transiently activated by elicitor treatment or illumination with UV light; however, a small set are expressed after exposure of etiolated hypocotyls to visible light. The divergent amino acid sequences of the predicted translation products of the EAS cDNAs and structural genes reported herein indicate the existence of multiple EAS isozymes in tobacco. This genetic polymorphism may position the enzymatic isoforms in separate temporal-, spatial-, or stress-specific regulatory networks to provide the optimum response to environmental cues.

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